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Community-Acquired Poliovirus Infection in Children with Primary Immunodeficiencies in Tunisia

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The global polio eradication program recommends the use of massive vaccination campaigns with live vaccine through National Immunization Days (NIDs) to displace the wild virus from the community. Immunodeficient patients may be indirectly infected and become chronic excretors and potential reservoirs of polioviruses, a concern for the posteradication era. This prospective study aimed to assess the risk of community-acquired infection of immunodeficient patients following NIDs, the dynamics of viral excretion and the genetic variation of excreted viruses. Sixteen children with various primary immunodeficiencies, who did not receive the vaccine during the campaign, were investigated. Stool samples were collected weekly, shortly after the NIDs, during at least 3 months, and were processed for viral isolation. Isolates were characterized by three intratypic differentiation methods and partial sequencing of the VP1/2A region. Polioviruses were detected in 4 out of 16 patients (serotype 1 in 3 patients and serotype 3 in 1 patient). Sequencing revealed more than 99% homology with homotypic Sabin strains, suggesting recent infection. Duration of viral excretion ranged from 1 to 7 weeks. Nine out of eleven isolates from the three poliovirus serotype 1-infected patients disclosed a non-Sabin-like phenotype by enzyme-linked immunosorbent assay and had recurrent mutations within or close to the neutralizing antigenic sites. In summary, the risk of secondary infection in immunodeficient patients is within the range previously reported for the general population. Although none of the four infected patients developed prolonged viral excretion, particular viral variants were selected and may be of epidemiological significance.

In 1988, the World Health Assembly committed the World Health Organization (WHO) to global eradication of poliomyelitis by the year 2000 (34). The global eradication program has emphasized the use of oral poliovirus vaccine (OPV), considering its numerous advantages, compared to the inactivated vaccine, i.e., its low cost, the logistic ease of its administration, and its ability to induce local gut immunity. During the last decade, the use of OPV was extended through mass campaigns which proved to be highly effective in interrupting wild virus transmission. This strategy aims to displace circulating wild polioviruses with vaccine-derived strains through extensive and synchronized national immunization days (NIDs). Once wild polioviruses are eradicated, vaccine-derived strains may then rapidly disappear from the community after cessation of immunization with OPV. Studies conducted in Cuba, where OPV is exclusively delivered through mass campaigns, demonstrated that OPV strains become undetectable, in young children and in the environment, 3 months after the last immunization round (24, 26). These features are certainly due to the limited survival of polioviruses in the environment and to the absence of long-term carrier state for polioviruses in immunocompetent individuals (1, 10). In contrast, individuals with immunodeficiencies may excrete polioviruses for several months and even years. In addition to the enhanced risk of emergence of a neurovirulent revertant causing paralytic disease in those patients (16, 19), the long-term excretion of polioviruses may constitute a potentially protracted source of infection in the posteradication era (13, 19). Recent findings, in Egypt (36), the Dominican Republic, and Haiti (27) demonstrated that OPV strains that reverted to neurovirulence are capable of sustained circulation, a characteristic of wild-type viruses. Therefore, a better understanding of the mechanisms and epidemiological situations favoring the circulation and transmission of OPV strains may shed light on the most appropriate vaccination strategies in the posteradication era.

In line with the WHO's strategy for eradicating poliomyelitis, a two-round NID was conducted in October and November 1996 in Tunisia. All children under 5 years received two doses of OPV. The present study was conducted in 16 children with well-characterized primary immunodeficiencies (4), thus excluded from OPV vaccination during the NIDs, to assess the risk of community-acquired poliovirus infection in these patients and to study the dynamics of enteric viral excretion and the genetic variation of excreted viruses.

MATERIALS AND METHODS

Patients. Sixteen patients with various primary immunodeficiencies were investigated (Table 1). The definite diagnosis was established according to the International Union of Immunological Societies classification (17): X-linked agammaglobulinemia (X-LA) ($n = 4$), common variable immunodeficiency (CVID) ($n = 2$), X-linked hyper-immunoglobulin M syndrome (X-LHIGM) ($n = 5$), T-cell activation deficiency (TCAD, $n = 1$), idiopathic disseminated BCGitis ($n = 1$), and chronic granulomatous disease (CGD) ($n = 3$). Patient characteristics are listed in Table 1. The children lived in eight different districts of the

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TABLE 1. Clinical features and polio vaccination status of immunodeficient patients in this study[a]

Patient no.	Gender	Prior vaccination	Immunodeficiency	Age at diagnosis	Age at time of present study	Substitutive Ig therapy
1	M	OPV	X-LA	9 mo	2 yr	Yes
2	M	OPV	X-LA	12 mo	15 yr	Yes
3	M	None	X-LA	26 mo	6 yr	Yes
4	M	OPV	X-LA	7 yr	9 yr	Yes
5	M	OPV	CVID	5 yr	11 yr	Yes
6	M	OPV	CVID	2 yr	9 yr	Yes
7	M	OPV	X-LHIGM	10 yr	15 yr	Yes
8	M	OPV	X-LHIGM	11 yr	16 yr	Yes
9	M	OPV	X-LHIGM	15 mo	2 yr	Yes
10	M	IPV	X-LHIGM	8 yr	16 yr	Yes
11	M	OPV	X-LHIGM	7 yr	11 yr	Yes
12	M	OPV	Idiopathic BCGitis	30 mo	6 yr	No
13	F	OPV	TCAD	15 mo	3 yr	No
14	F	OPV	CGD	12 mo	13 yr	No
15	M	IPV	CGD	12 mo	6 yr	No
16	M	OPV	CGD	12 mo	13 yr	No

[a] Abbreviations: M, male; F, Female; IPV, inactivated polio vaccine; TCAD, T-cell activation deficiency; Ig, immunoglobulin.

country. None was given OPV during the two NID rounds (14 October and 16 November 1996). Thirteen had uneventfully received OPV during the first year of age, before their immunodeficiency was diagnosed. Ten patients were under substitutive intramuscular immunoglobulin therapy when the study was conducted.

Specimens. Fecal samples were collected at weekly intervals, during a period of 14 weeks, from 28 October 1996 (2 weeks after the first NID round) to 7 February 1997 (3 months after the second NID round) (Fig. 1). Ten patients had a complete set of samples (12 samples); patients 7, 8, and 3 had 11 samples each; patients 1 and 4 had 10 samples each; and patient 13 died from sepsis during the course of the study, with only 7 samples having been collected. Immediately after collection, specimens were stored at −20°C, and samples were transported to our laboratory, on dry ice, when the complete set of specimens for each patient was obtained. Patients from whom polioviruses were isolated from the stools were revisited 36 months later and three stool specimens were collected at 24-h intervals.

Virus isolation and serotyping. Fecal specimens were processed for virus isolation and identification using standard protocols recommended by the WHO (35). Briefly, stools were inoculated on RD (human embryo rhabdomyosarcoma) and HEp2-Cincinnati (epidermoid carcinoma, human larynx) cell lines. Specimens were considered negative if no cytopathic effect was detected 14 days after inoculation. For positive samples, isolated viruses were typed by seroneutralization of cytopathic effect using pools of antisera against polioviruses (Rijksinstituut voor Volkgezondheid en Milieuhygiëne, Bilthoven, The Netherlands).

Intratypic differentiation (ITD). In order to establish whether the poliovirus isolates were of vaccine or wild origin, they were tested by three methods recommended by the WHO for polio surveillance: (i) an enzyme-linked immunosorbent assay (ELISA) using cross-absorbed intratype-specific rabbit antisera (Po-Ab ELISA), allowing detection of antigenic differences between non-Sabin and Sabin-derived strains (15), (ii) a hybridization assay using riboprobes hybridizing specifically with the genome of vaccine-related isolates (8), and (iii) a reverse transcription-PCR amplifying a 480-nucleotide sequence in the VP1-2A region of the genome followed by digestion with restriction enzymes and analysis of restriction fragment length polymorphisms (RFLP), in comparison with the electrophoretic patterns of Sabin strains (3). All assays were performed as described by the respective authors.

Sequence analysis. Complete VP1 and partial 2A sequences (nucleotides 2480 to 3445) were determined. Amplification and sequencing assays used UC13 (nucleotides 3629 to 3648 5′-TAGTCATTAGCTTCCATGTA-3′) or UC1 (nucleotides 2861 to 2881, 5′-GAATTCCATGTCAAATCTAGA-3′) as reverse primers and UG19 (nucleotides 2870 to 2891, 5′-GACATGGAATTCACCTTTGTGG-3′) or UG1 (nucleotides 2402 to 2421, 5′-TTTGTGTCAGCGTGTAATGA-3′) as upstream primers. The amplified DNAs were purified by the Qiaex PCR purification kit (Qiagen) and then sequenced in the forward and reverse directions, using an automated sequencer (ABI PRISM 377-3.0). Analysis of VP1/2A sequences used DNA Strider 1.2 and Clustal V programs.

RESULTS

Poliovirus excretion. A total of 179 stool specimens obtained from the 16 immunodeficient patients were analyzed. Polioviruses were isolated from 13 stool specimens obtained from four patients living in four different districts of the country. Patients 7 and 3 suffered X-LHIGM, patient 6 had CVID, and patient 12 suffered idiopathic BCGitis. Three patients excreted poliovirus serotype 1, and one patient excreted poliovirus serotype 3 (Fig. 1): patient 9 excreted poliovirus type 1 in every stool cultured over a period of 7 weeks; patient 7 had one poliovirus serotype 1-positive stool, a 5-week interval with negative stools, and then another poliovirus serotype 1-positive stool; patient 6 had only one sample positive for serotype 1. Patient 12 had two consecutive samples positive for poliovirus type 3 (Fig. 1). Thus, among the four infected patients, patients 6 and 12 seem to have rapidly stopped poliovirus excretion, whereas for patients 7 and 9, poliovirus excretion lasted at least 7 weeks. In patient 7 the nondetection of polioviruses in stool samples 3 to 8 may be explained by the intermittence of poliovirus shedding and the relatively long interval of our sampling. Finally, to assess whether long-term viral excretion could have been maintained in these patients as previously reported (5, 19, 23), the four infected patients were revisited 36 months later. The three stools collected at 24-h intervals were negative, a highly sensitive criterion indicating the absence of virus multiplication (14).

ITD. All poliovirus isolates were analyzed by three ITD methods. Probe hybridization and PCR-RFLP analysis confirmed the vaccine-derived origin of all poliovirus type 1 (n = 11) and poliovirus type 3 (n = 2) isolates: thus, a positive signal was obtained with homotypic Sabin-specific probes (Fig. 2) and RFLP analysis of the VP1/2A region using *Hae*III, *Hpa*II, and *Dde*I restriction enzymes, gave a pattern similar to that of corresponding Sabin strains (Fig. 3). Po-Ab ELISA also confirmed the vaccine-derived origin of the two poliovirus type 3 isolates; however, it disclosed a strikingly aberrant phenotype for 9 out of the 11 poliovirus type 1 isolates, excreted by

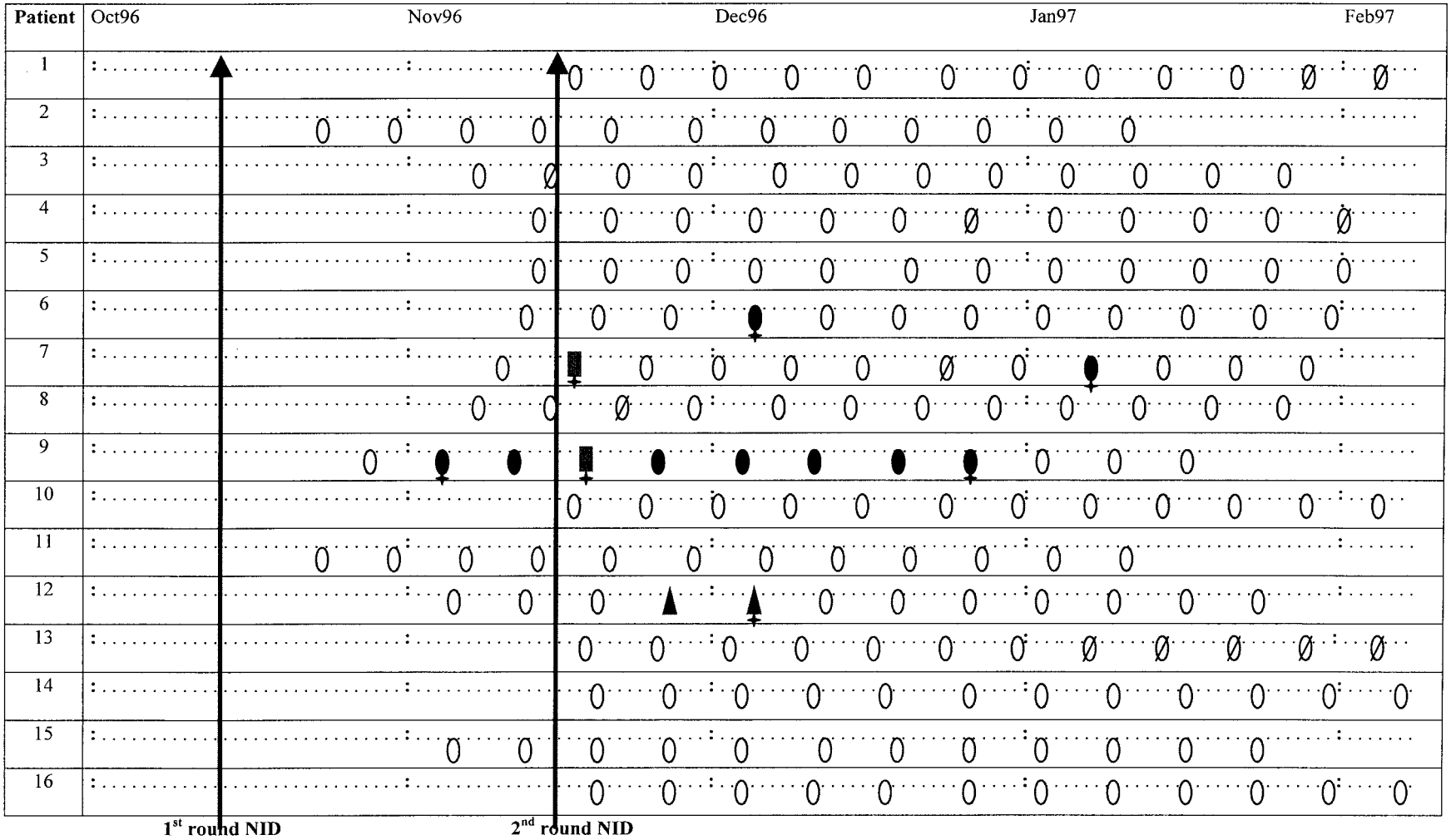

FIG. 1. Timing of stool collection, virus excretion and strains characteristics. Symbols: open oval, stool collected, enterovirus negative; oval with slash, stool not collected; rectangle, poliovirus type1, SL by Po-Ab ELISA; filled oval, poliovirus type1, NSL by Po-Ab ELISA; triangle, poliovirus type 3, SL by Po-Ab ELISA, plus sign, isolate sequenced.

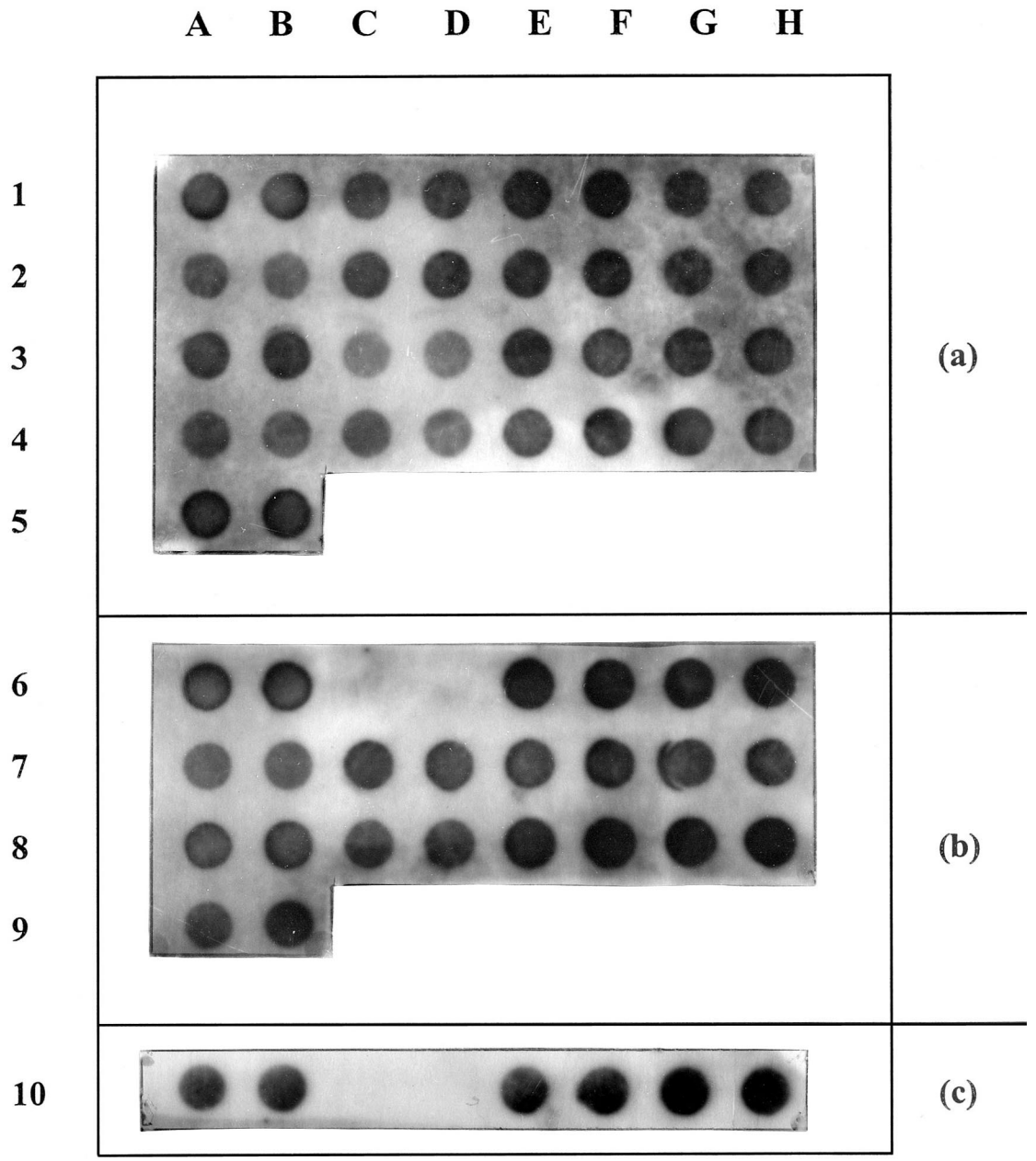

FIG. 2. Intratypic differentiation using Sabin-specific riboprobe hybridization. Hybridization with the enterovirus-specific probe (a), Sabin 1-specific probe (b), and Sabin 3-specific probe (c). Samples were blotted to the nylon membrane and duplicates were used for each probe. The Sabin 1 control reacted positively with the enterovirus-specific probe (reactions A1 and B1) and the Sabin 1 specific probe (reactions A6 and B6). Wild poliovirus type 1 control reacted positively with the enterovirus-specific probe (reactions C1 and D1) and did not react with the Sabin 1-specific probe (reactions C6 and D6). The Sabin 3 control reacted positively with the enterovirus-specific probe (reactions C4 and D4) and the Sabin 3-specific probe (reactions A10 and B10). Wild poliovirus type 3 control reacted positively with the enterovirus-specific probe (reactions E4 and F4) and did not react with the Sabin 3-specific probe (reactions C10 and D10). All strains isolated from the patients reacted with the enterovirus-specific probe and the Sabin-specific probe from the same serotype: 7-2 (reactions E1, F1, E6, and F6), 7-9 (reactions G1, H1, G6, and H6), 9-2 (reactions A2, B2, A7, and B7), 9-3 (reactions C2, D2, C7, and D7), 9-4 (reactions E2, F2, E7, and F7), 9-5 (reactions G2, H2, G7, and H7), 9-6 (reactions A3, B3, A8, and B8), 9-7 (reactions C3, D3, C8, and D8), 9-8 (reactions E3, F3, E8, and F8), 9-9 (reactions G3, H3, G8, and H8), 6-4 (reactions A4, B4, A9, and B9), 12-4 (reactions G4, H4, E10, and F10), 12-5 (reactions A5, B5, G10, and H10). The film image was generated using Adobe Photoshop software.

patients 7, 9, and 6, which exhibited a non-Sabin-like (NSL) phenotype (Fig. 1 and 4).

Partial sequencing. The nucleotide sequence of the entire VP1 region and part of the 2A region (nt 2480 to 3386) was determined for six isolates of serotype 1 (7-2, 7-9, 6-4, 9-2, 9-4, and 9-9) and one isolate of serotype 3 (12-5); the isolates are designated by the patient's number followed by the specimen number according to the timing of stool collection (Fig. 1).

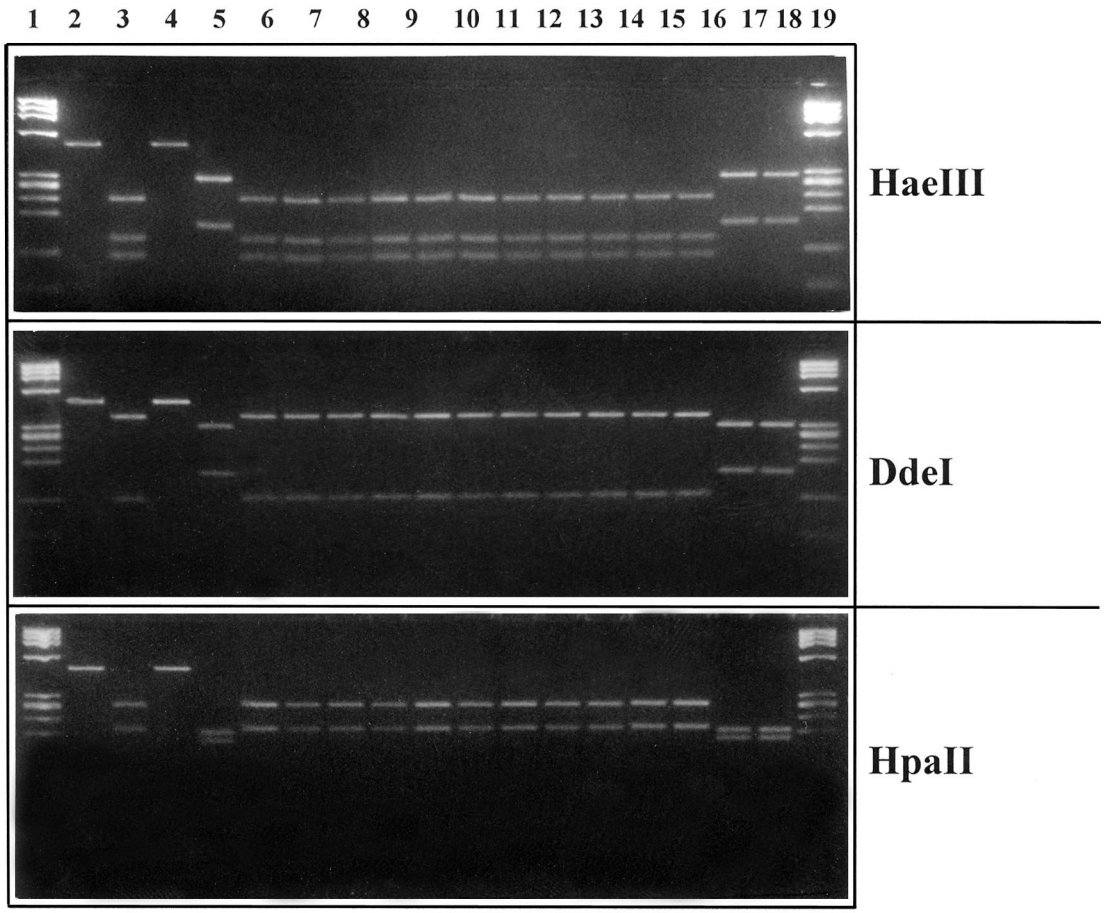

FIG. 3. Intratypic differentiation by PCR-RFLP analysis. Ethidium bromide-stained 3% agarose gel showing the RFLP patterns of the different patient isolates and those of Sabin 1 and Sabin 3 strains. The panels show PCR products digested by *Hae*III, *Hpa*II, and *Dde*I. Lanes1 and 19 correspond to the molecular weight marker (PhiX digested with *Hae*III); lanes 2 and 4 correspond to nondigested amplification products of Sabin 1 and Sabin 3, respectively; lanes 3 and 5 correspond to the digested amplification products of Sabin 1 and Sabin 3, respectively; and lanes 6 to 18 correspond to the patient poliovirus isolates (7-2, 7-9, 9-2, 9-3, 9-4, 9-5, 9-6, 9-7, 9-8, 9-9, 6-4, 12-4, and 12-5, respectively). The gel image was generated using Adobe Photoshop software.

Comparison with corresponding regions in Sabin strains revealed a nucleotide identity ranging from 99.3 to 99.7%. Two to six point mutations per strain were observed throughout the sequenced fragment. Among the six isolates of serotype 1, four (7-9, 6-4, 9-2, and 9-9) counted among the nine isolates that exhibited an NSL phenotype by Po-Ab ELISA, whereas two isolates (7-2 and 9-4) had a Sabin-like (SL) phenotype. Coding mutations within or very close to the neutralizing antigenic (NAg) sites I and III (positions 2748, 2750, 2769, and 3349) were identified in the four isolates with aberrant NSL phenotype but not in the two isolates with SL phenotype. Mutations at positions 2769 and 3349 within NAgI and NAgIII sites, respectively, occurred in three out of the four strains, those at positions 2748 or 2750, affecting codon 90 of VP1 (the last codon before the NAgI site) occurred in all four strains (Fig. 5). Isolate MA5 of serotype 3 exhibited only four mutations, resulting in two amino acid changes outside the neutralizing antigenic sites. The overall nucleotide homology with the Sabin 3 strain was 99.5%.

DISCUSSION

Although immunodeficiencies are listed as a contraindication for receiving OPV, patients with these clinical conditions may occasionally receive the poliovirus live vaccine before their immunodeficiency is diagnosed and/or may be infected with OPV strains excreted by a vaccinee or circulating within the community. When infection with vaccine strains occurs in such patients, protracted virus replication can take place and may last as long as 10 years (19). Beside the risk of developing paralysis due to the emergence of neurovirulent revertants, the issue of long-term excretors was recently raised as a major concern for the polio global eradication program, these patients being a potential reservoir of polioviruses in the poster-adication era (13, 18, 33). Two recent studies reported prolonged replication of vaccine-derived polioviruses without paralysis in immunodeficient patients after receiving OPV (5, 23). In line with this concern, we explored the potential of community-transmitted polio infection to generate long-term excretors in children with primary immunodeficiencies.

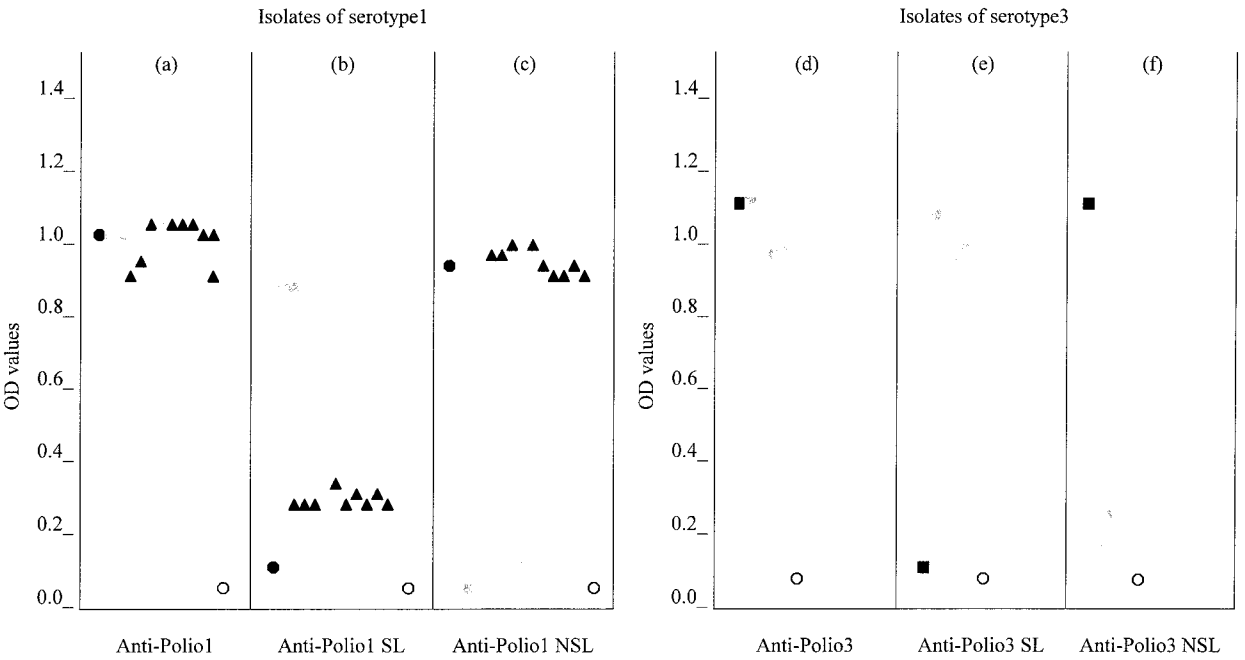

FIG. 4. Intratypic differentiation by Po-Ab ELISA. Symbols: open circle, background; filled circle, poliovirus type1 NSL control (Mahoney); shaded circle, poliovirus type 1 SL control (Sabin 1); filled square, poliovirus type 3 NSL control (Finland); shaded square, poliovirus type3 SL control (Sabin 3); filled triangle, patients' isolates with NSL phenotype; shaded triangle, patients' isolates with SL phenotype. The figure shows the reactivity of controls and patients' isolates with unabsorbed antisera specific to all polioviruses type1 (a) or type3 (d) and with cross absorbed antisera specific to SL-polio1 (b), NSL-polio1 (c), SL-polio3 (e) and NSL-polio3 (f) viruses.

Since the beginning of the era of vaccination against poliomyelitis, the property of OPV strains to spread from vaccinees to susceptible individuals has been considered advantageous, as it results in immunization of individuals who may have escaped vaccination. Recent studies provided indirect evidence of vaccine virus spread to nonvaccinated individuals in the general population, either during mass vaccination campaigns (24, 28) or secondary to routine vaccination with OPV (7); the estimated rates of secondary transmission range from 9 to 65%. In our study, 4 out of 16 patients (25%) were infected; this rate of horizontal transmission to immunocompromised patients does not indicate higher susceptibility to polio infection. Molecular analysis of excreted polioviruses confirmed that they resulted from a recent infection, most likely during NIDs. Indeed, during replication of vaccine viruses in individuals and their transmission from person-to-person in the community, mutations will accumulate. The duration of circulation or excretion of a vaccine-derived poliovirus strain can be estimated by determining the extent of its divergence from the original Sabin strain, the rate of nucleotide changes per year, at synonymous third-base codon position, in the VP1/2A region of the genome, being approximately 3.3% (19, 23). In our study, isolates excreted by all four infected patients were very close to the original Sabin strains, with less than 1% nucleotide divergence, including coding mutations.

Most studies reporting vaccine-associated paralytic poliomyelitis concerned patients with agamma- or hypogammaglobulinemia (29, 37). It is likely that the absence of specific antibodies to polioviruses in these patients is responsible for the lack of viral neutralization during the viremic phase, which precedes neurological localization. On the other hand, prolonged virus replication and excretion without paralysis may be the consequence of defects in other antiviral effector mechanisms. T-cell immunodeficiencies have been reported to be associated with persistence of other enteric viral infections (32). In our series, two polio-infected patients were affected with X-LHIGM and one was infected with CVID. These two immunodeficiencies are basically characterized by a T-cell defect, which not only impairs antibody production by B cells but also affects T-cell effector functions. Thus, X-LHIGM is due to mutations in the CD40 ligand (CD40L) gene, normally expressed by activated T cells (9). On the other hand, a subset of CVIDs is also a result of defective expression of CD40L (12). We have confirmed that patients 7 and 9 totally lacked CD40L expression, whereas patient 6 had a decreased expression of this molecule (unpublished data). Interestingly, several reports recently stressed the critical role of CD40-CD40L interactions in the generation of functional CD4 and CD8 T cells and in the amplification of the mucosal CD8-T-cell responses (2, 6, 20). In our study, patient 6 rapidly stopped poliovirus excretion; the two other patients (patients 7 and 9) excreted the virus during at least 7 weeks. A literature review on the duration of poliovirus excretion showed that fecal excretion of vaccine viruses continues beyond the fifth week in less than 20% of immunized individuals (1). Although the excretion period of at least 7 weeks observed in the two patients affected with X-LHIGM may be considered to be slightly prolonged, the fact that these patients ultimately cleared viral infection suggests the intervention of CD40L-independent viral T-cell control mechanisms, as recently reported in experimental models (21). Substitutive immunoglobulin therapy prescribed to the three patients may have contributed to the clearance of the virus. It

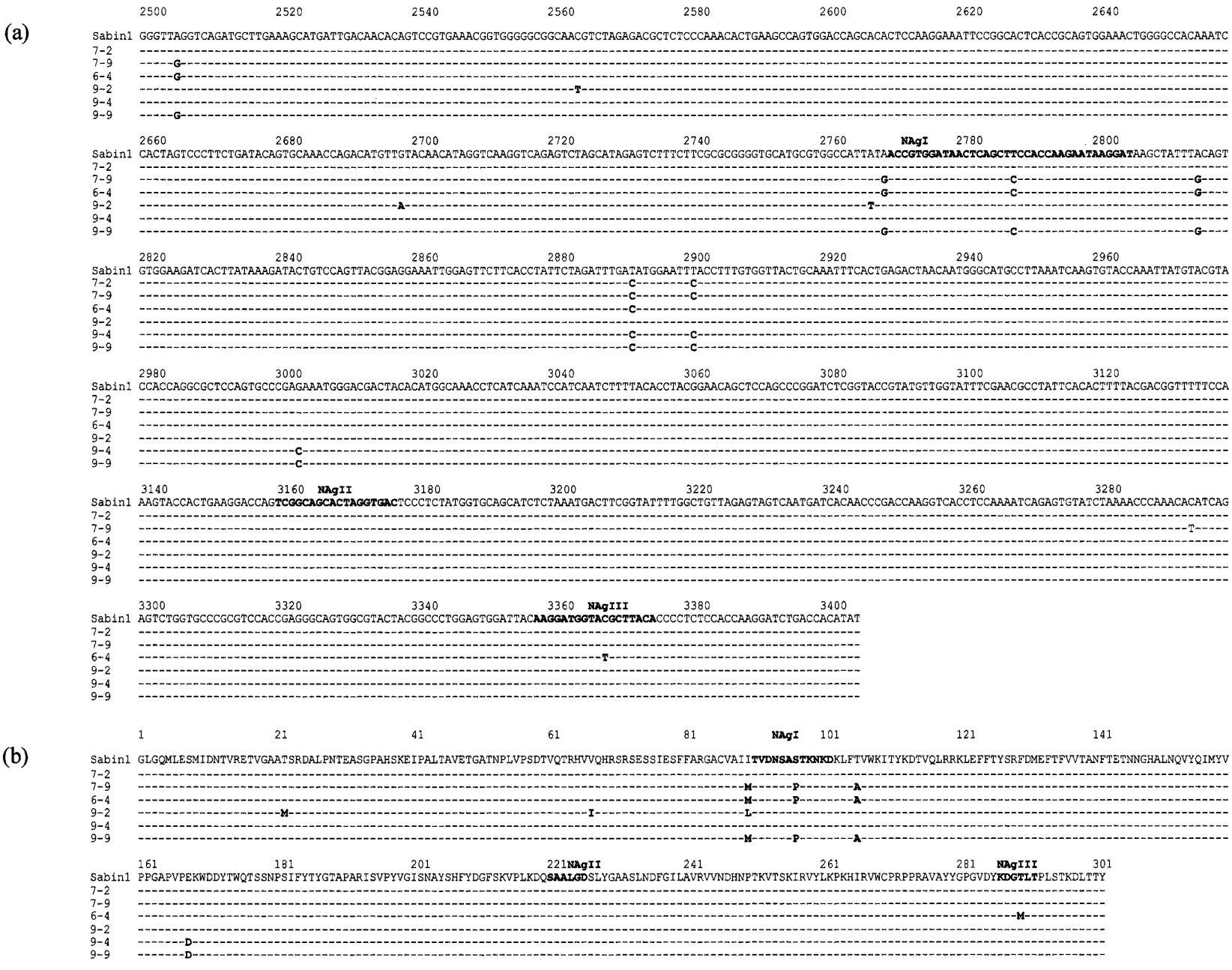

FIG. 5. Partial nucleotide (a) and deduced amino acid (b) sequences of serotype 1 isolates compared to the reference Sabin 1 strain. Numbering of nucleotides was done according to the method of Toyoda et al. (30). The NAgI to NAgIII are shown in boldface type. Capsid amino acid residues are numbered starting from residue 1 of VP1.

has been previously suggested that patients treated with immunoglobulins could be more resistant to infection and only few of them become long-term excretors (22). Finally, the fourth infected patient (patient 12) had idiopathic disseminated BCGitis, the molecular basis for which is heterogeneous, involving either a T-cell or a macrophage defect (11). Although the specific molecular defect in this patient is currently unknown, the kinetics of viral excretion indicates a rapid control of poliovirus replication.

Previous studies reported immunodeficient patients who converted to long-term viral excretors shortly after receiving OPV vaccine, thus corresponding to massive infecting doses (5, 19, 23). The fact that the patients investigated herein were indirectly infected from the community likely implies lower infecting doses, lower risk of establishing infection and better chance to rapidly clear the virus from the gut.

Despite the very low nucleotide divergence from the original Sabin strain, 9 (out of 11) poliovirus type 1 isolates detected in three (out of four) infected patients exhibited an aberrant NSL phenotype by Po-Ab ELISA ITD test. In contrast, probe hybridization and PCR-RFLP indicated clearly a vaccine-derived origin. Such discordances between genotype-based and phenotype-based ITD methods do exist but have been rarely noticed in field vaccine-derived strains isolated from immunocompetent vaccinees, paralytic patients, or their healthy contacts (25). Sequencing of the VP1/VP2 genomic regions of four isolates with the aberrant phenotype revealed coding mutations within or very close to the neutralizing antigenic sites I or III, similar to those reported in other vaccine derived strains of serotype1 exhibiting the same NSL characteristics by Po-Ab ELISA (19, 25). The fact that three out of four passively infected patients excreted viruses with this aberrant phenotype is very interesting. These vaccine-derived strains may have recovered higher growth or transmissibility potentials, which allowed their selection under the specific pressures which may exist in the gut of immunodeficient patients. Our results do not allow us to conclude whether the patients had picked up from the community variants which had already mutated or whether they generated these variants during replication in their gut. Interestingly, the recently reported vaccine-derived polioviruses that circulated in Egypt and caused paralytic cases of polio also expressed an NSL aberrant phenotype by ELISA (36). It should be interesting to evaluate whether such discordance between phenotypic and genotypic characteristics correlates with higher transmissibility of OPV-derived strains. These results further stress the importance of the systematic use, as already recommended by WHO (31, 35), of two different ITD methods, one based on genetic and the other on antigenic characterization of the isolates to track down such variants.

This study helps to clarify some of the issues raised by the use of massive vaccination campaigns. The risk of secondary infection does not appear to be higher in primary immunodeficient patients than in immunocompetent patients. Such secondary infections are unlikely to lead to protracted viral excretion, possibly due to the low infecting doses in person-to-person transmission compared to direct administration of OPV. Appropriate coverage by substitutive immunoglobulin therapy, where indicated, during NIDs may also help to rapidly clear the viral infection. Despite the lack of long-term excretors in our series, one might be concerned by the unusual viral variants which may be selected by these patients and may have enhanced growth and/or transmissibility potentials and, thus, be of epidemiological significance.

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